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Human Adenylyl Cyclase Type 7 Contains Polymorphic Repeats in the 3′ Untranslated Region: Investigations of Association With Alcoholism

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Platelet adenylyl cyclase activity has been proposed as a trait marker for alcoholism [Tabakoff et al. (1988): N Engl J Med 318:134–139; Parsian et al. (1996): Alcohol Clin Exp Res 20:745–751]. Human adenylyl cyclase type 7 (ADCY7) is a member of the adenylyl cyclase gene family, and it may be the major form of adenylyl cyclase expressed in human platelets. The published cDNA sequence of ADCY7 indicated the presence of potentially polymorphic regions in the 3′ untranslated region of ADCY7. PCR techniques combined with fluorescently labeled primers were used to amplify two separate tetranucleotide repeat regions [$(AACA)_n$] in the 3′ untranslated region of ADCY7 from the genomic DNA of 62 unrelated individuals. The upstream $(AACA)_4$-repeat was not polymorphic. Five different genotypes were found in the downstream $(AACA)_{5–7}$ tetranucleotide repeat region. We also tested the association of the tetranucleotide polymorphism to alcohol dependence. When 30 alcoholic and 17 control individuals were compared, no difference was found in the ADCY7 tetranucleotide polymorphism between alcohol-dependent and control groups. Nevertheless, to our knowledge these are the first polymorphisms reported in an adenylyl cyclase gene. Adenylyl cyclases are important receptor-G protein-coupled effectors and are involved in numerous neuronal functions in the central nervous system. Whether variations in ADCY7 and possible variations in other members of this gene family are underlying other psychiatric disorders remains to be studied. ***Am. J. Med. Genet. 74:95–98, 1997.***

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INTRODUCTION

Adenylyl cyclases (EC 4.6.1.1) are a family of G-protein-regulated, plasma membrane-bound enzymes that catalyze formation of adenosine 3′,5′-cyclic monophosphate (cyclic AMP). Cyclic AMP, in turn, activates protein kinase A in different cell types. Previous studies have shown that alcohol-dependent individuals have significantly lower platelet and lymphocyte adenylyl cyclase activity compared to controls [Diamond et al., 1987; Tabakoff et al., 1988; Lex et al., 1993; Waltman et al., 1993; Parsian et al., 1996], and recent studies have suggested that the adenylyl cyclase catalytic unit rather than G-proteins may be the major contributor to low platelet adenylyl cyclase activity in alcohol-dependent individuals [Parsian et al., 1996]. Genetic studies have also provided evidence for familial transmission of platelet adenylyl cyclase activity [Devor et al., 1991; Lex et al., 1993; Saito et al., 1994], and for a major gene effect transmitted as a Mendelian codominant trait [Devor et al., 1991]. These data prompted us to study the possible genetic variation of an adenylyl cyclase isoform (ADCY7) that may be the major adenylyl cyclase isoform in human platelets.

The adenylyl cyclase gene family currently consists of nine different gene products (ADCY1–ADCY9) that have unique functional properties [Iyengar, 1993; Krupinski et al., 1992; Paterson et al., 1995; Taussig and Gilman, 1995]. Lack of type-specific adenylyl cyclase antibodies has so far prevented the direct analysis of platelet adenylyl cyclase isoforms at the protein level, but Nanoff et al. [1994] demonstrated, using G-protein-reconstitution assays, that the pattern of adenylyl cyclase stimulation in human platelet membranes is characteristic of the adenylyl cyclase type 2 subfamily. Our data from the human erythroleukemia (HEL) cell line, which has been widely used as a model for platelet studies [Martin and Papayannopoulou, 1982; Papayannopoulou et al., 1983; Brass et al., 1991], suggest that

ADCY7 mRNA is the major adenylyl cyclase form in this cell line [Hellevuo et al., 1993]. Functionally, ADCY7 can be characterized as a member of the type 2 adenylyl cyclase subfamily [Hellevuo et al., 1995b; Yoshimura et al., 1996], and such data suggest that ADCY7 may be the major adenylyl cyclase isoform in human platelets. ADCY7 is also expressed in the cerebellum [Hellevuo et al., 1995b]. Interestingly, male individuals with positive family history for alcoholism show diminished sensitivity to ethanol in tests that measure standing steadiness [Schuckit, 1994]. Cerebellar Purkinje cells, which are the major efferent cells of the cerebellum, have an important role in motor functions such as standing steadiness, and ethanol has been shown to inhibit cerebellar Purkinje cell firing through its potentiation of GABA actions at the $GABA_A$ receptor. This effect of ethanol, however, has been shown to require the concomitant activation of adenylyl cyclase [Lin et al., 1991]. An inherently lower activity of a specific adenylyl cyclase, such as ADCY7 in the Purkinje cells, could diminish the sensitivity of the Purkinje cell $GABA_A$ receptors to the actions of ethanol. Consequently, an individual's sensitivity to the motor-impairing effects of ethanol may be related to the characteristics of adenylyl cyclase in that individual's brain and other organs.

In this paper we describe a tetranucleotide repeat polymorphism in the 3′ untranslated region of human ADCY7. To our knowledge, this is the first report on a polymorphism in the adenylyl cyclase gene family. Secondly, we present our initial data indicating that this ADCY7 polymorphism is not associated with a diagnosis of alcohol dependence.

MATERIALS AND METHODS

The alcohol-dependent subjects for this study were recruited from the Colorado Psychiatric Hospital, Denver Veterans Administration Medical Center, Addiction Research Treatment Service, Arapahoe House Treatment Facility, and the community in the Denver, Colorado area. Control subjects were recruited from University of Colorado personnel, University Hospital staff, and the community. Altogether, 62 unrelated individuals (51 Caucasians, 4 Native Americans, 4 African-Americans, and 3 Asians) were recruited for this study. All subjects were required to abstain from alcohol for at least 7 days before the interview and the blood draw. Subjects with bipolar disorder or a major psychotic disorder were excluded from this study on the basis of a screening tool which was derived from the High Mood Section of the NIAAA Alcohol Use Disorders and Disabilities Schedule (AUDADIS) [Grant et al., 1995] and the psychosis section of the Semi-Structured Assessment for the Genetics of Alcoholism (SSAGA) [Buckholz et al., 1994]. After giving written consent, the subjects completed a Beck Depression Inventory [Beck et al., 1961]. The full structured interview was conducted by one of the authors (J.A.M.). The interview included the WHO/ISBRA (World Health Organization/International Society for Biomedical Research on Alcoholism) Interview Schedule and the Low Mood and Behavior portions of AUDADIS. The WHO/ISBRA instrument allowed DSM-III-R, DSM-IV, and ICD-10 diagnoses of alcohol and drug dependence and abuse. The AUDADIS allowed measures of alcohol consumption and drug use. The Low Mood and Behavior portion of AUDADIS instrument allowed DSM-III-R, DSM-IV and ICD-10 diagnoses of major depressive disorder and dysthymia. Genomic DNA was obtained from all 62 individuals who participated in this study, and it was used to assess the nature of tetranucleotide polymorphisms in ADCY7. Based on interviews of the 62 individuals, 30 (23 Caucasian, 4 Native American, and 3 African-American) were diagnosed as alcohol-dependent, 15 individuals (all Caucasian) were diagnosed as depressed, and 17 control individuals (13 Caucasian, 3 Asian, and 1 African-American) were judged neither alcohol-dependent nor depressed. The depressed individuals were excluded from the association analysis of the polymorphism and alcohol dependence.

Peripheral blood was collected (20 ml in 0.15% w/v EDTA) and fractionated immediately after collection, as described earlier [Tabakoff et al., 1988]. The lymphocyte fraction was frozen at −70°C until DNA extraction. Genomic DNA was extracted from the lymphocytes using the Analytical Genetic Testing Center's (Denver, CO) Super Quick-Gene DNA Isolation Kit, according to the kit protocol.

Two sets of primers were designed from the available ADCY7 (Genbank D25538) sequence using PRIMER software [Lincoln et al., 1991]. The upstream region 1 primers (sense 5′CACTGTGCTGTGCT CACATA3′, and antisense 5′TATCTGAGCACAGCACAGTG3′) flanking $(AACA)_4$ were expected to yield a 209-base pair PCR product. The downstream region 2 primers (sense 5′TTCTCCATGGGTCAAGGACT3′, and antisense 5′ATGATTTGAGGTGGTGCATG3′) flanking $(AACA)_6$ were expected to yield a 199-base pair product. The primers were synthesized by use of an Applied Biosystem (ABI) (Foster City, CA) 394 DNA Synthesizer, and the 5′ end of the sense primers was labeled with ABI's blue fluorescent phosphoramidite, 6-FAM amidite. All primers were column-purified using ABI's Oligonucleotide Purification Cartridge.

PCR was performed on a Perkin-Elmer Gene Amp PCR System 9600 thermocycler (Perkin-Elmer, Foster City, CA). Only one region was amplified per reaction. The 15-μl reaction volume contained 40 ng of genomic DNA, 100 ng of each primer, 250 μM of each dNTP, and 0.4 U of AmpliTaq polymerase in standard 1 × GeneAmp buffer. All reagents were from Perkin-Elmer (Foster City, CA). The cycling conditions were as follows: 94°C for 4 min, 35 cycles of 94°C for 15 sec, 55°C for 1 min 15 sec, and 72°C for 1 min 15 sec, followed by an extension at 72°C for 10 min and a 4°C soak. Negative controls (no template) were included in every set of amplification reactions. Every sample of DNA underwent two independent PCR amplification reactions for both regions to verify allele lengths.

After PCR, aliquots of the samples were mixed with ABI's fluorescent Genescan-500 ROX internal lane standard and electrophoresed on ABI's Model 373 Sequencer. Fluorescence data was digitized and transmitted to a Macintosh computer equipped with GENESCAN 672, version 3.0 software. The PCR product lengths were determined based on internal lane standards using the Linear Southern Curve option of the analysis software.

After assessment of the genotypes in region 2 of ADCY7 (Table I), 30 alcoholic (23 Caucasian, 4 Native

TABLE I. Genotypes of the Two Tetranucleotide Repeat Regions in the 3′ Untranslated Region of ADCY7 in 62 Unrelated Individuals

Region 1 genotypes			Region 2 genotypes		
$(AACA)_4/(AACA)_4$	62/62	(100%)	$(AACA)_5/(AACA)_5$	0/62	(0%)
			$(AACA)_5/(AACA)_6$	7/62	(11%)
			$(AACA)_5/(AACA)_7$	4/62	(6%)
			$(AACA)_6/(AACA)_6$	26/62	(42%)
			$(AACA)_6/(AACA)_7$	19/62	(31%)
			$(AACA)_7/(AACA)_7$	6/62	(10%)

American, and 3 African-American) and 17 control (13 Caucasian, 3 Asian, and 1 African-American) individuals or, to match for ethnicity, the Caucasian individuals (23 alcohol-dependent and 13 control individuals) were tested in an association analysis (Table II and Results). Statistical analysis was performed using chi-square analysis (SigmaStat Version 2.0 software).

RESULTS AND DISCUSSION

Of the two tetranucleotide repeat regions, region 1 [$(AACA)_4$] was not polymorphic (Table I). Region 2 $(AACA)_n$ displayed five allele combinations, with 26 of 62 individuals (42%) being homozygous for the $(AACA)_6$ allele (Table I). Six individuals (10%) were homozygous for $(AACA)_7$, and 19 individuals (31%) were heterozygous for $(AACA)_6/(AACA)_7$. Seven (11%) and 4 (6%) individuals were heterozygous for $(AACA)_5/(AACA)_6$ and $(AACA)_5/(AACA)_7$, respectively (Table I).

It is of interest that of the nine adenylyl cyclases genes, all adenylyl cyclase isoforms which have been mapped so far (ADCY1–ADCY8) are located on different chromosomes [Stengel et al., 1992; Haber et al., 1994; Hellevuo et al., 1995a], suggesting independent regulation of transcription. Since ADCY7 is a likely candidate for the adenylyl cyclase expressed in human platelets [Hellevuo et al., 1993; Nanoff et al., 1994], it may be responsible for the low adenylyl cyclase activity in platelets of alcohol-dependent individuals [Parsian et al., 1996]. When we tested the relationship between the 3′ untranslated tetranucleotide repeats of ADCY7 and alcohol dependence, chi-square analysis of the data indicated that there is no significant difference between the alcohol-dependent and control genotypes when 30 (23 Caucasian, 4 Native American, and 3 African-American) alcohol-dependent individuals and 17 (13 Caucasian, 3 Asian, and 1 African-American) control individuals were used in the analysis (chi-square 0.212, df = 4) (Table II). Additionally, assessment of the Caucasian individuals separately also produced no significant differences in genotype between alcohol-dependent and control subjects (chi-square 1.606, df = 4). The power of our analysis, however, was below the desired power for definitive conclusions to be drawn, due to small number of individuals in certain groups. In addition, for a more accurate analysis one may also need to define more homogenous subgroups of alcohol-dependent individuals [Cloninger, 1987; Gilligan et al., 1987; Brown et al., 1994].

Each adenylyl cyclase form, including ADCY7, has a unique expression pattern in brain tissue [Cali et al., 1994; Cooper et al., 1994; Hellevuo et al., 1995b] and in peripheral tissues [Iyengar, 1993; Krupinski et al., 1992]. In the central nervous system, adenylyl cyclases have important roles in synaptic transmission and in other neuronal functions. It will be of interest to see whether the tetranucleotide polymorphisms we have noted in the 3′ untranslated region of ADCY7 and possible polymorphisms in other members of this gene family are predictive of or determine variation in normal cellular adenylyl cyclase-mediated signal transduction, and whether certain polymorphic forms of ADCY7 or other adenylyl cyclases are related to any psychiatric disorders.

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TABLE II. ADCY7 $(AACA)_{5–7}$ Alleles: Genotypes of Alcohol-Dependent and Control Individuals*

		$(AACA)_n$ genotypes				
Group	Total N	$(AACA)_5/(AACA)_6$	$(AACA)_5/(AACA)_7$	$(AACA)_6/(AACA)_6$	$(AACA)_6/(AACA)_7$	$(AACA)_7/(AACA)_7$
Alcohol dependent	30	4 (3 Caucasian, 1 Native-American)	1 (1 Caucasian)	12 (10 Caucasian, 2 Native-American)	9 (6 Caucasian, 1 Native-American, 2 African-American)	4 (3 Caucasian, 1 African-American)
Control	17	2 (2 Caucasian)	1 (1 Caucasian)	7 (3 Caucasian, 1 African-American, 3 Asian)	5 (5 Caucasian)	2 (2 Caucasian)

* N, number of individuals. Chi-square = 0.212 with 4 degrees of freedom. No statistical significance was observed, i.e., frequencies for the noted alleles did not differ between alcohol-dependent and control individuals.

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